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Secondary traits related to kernel setting in transgenic maize hybrids at low and high plant densities and their potential use in breeding programs

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Abstract

Maize (Zea mays. L) traditional breeding presents limitations when selection is performed by adaptation to suboptimal conditions. One strategy to achieve greater efficiency in these environments is to include measurements of secondary traits related to the main grain yield component, i.e. kernel number per plant (KNP), which is associated to other three secondary traits: plant growth rate during the critical period (PGRcp) for kernel setting, biomass partitioning index to the ear during this period (PIcp) and the efficiency of the ear to set kernels or reproductive efficiency (RE). Phenotypic variations in these KNP related traits were evaluated considering: i) plant densities, and ii) simple and staked transgenic maize hybrids. The objective of this study was to identify the contribution of these secondary traits to KNP in six modern single-cross transgenic hybrids: the simple and staked transgenic versions of DK747 and DK190 (Bt: 747MG and 190MG; RR: 747RR and 190RR; and Bt-RR: 747MGRR and 190MGRR) cultivated at low and high plant densities. For this purpose, KNP and secondary traits were recorded at the individual plant level and data set was analyzed using multivariate analysis techniques. At low plant density, PGRcp was the critical trait for kernel setting in all hybrids but PIcp or RE also contributed to KNP of the DK747 group and the D190 group, respectively. At high plant density, a differential contribution of secondary traits to KNP was recorded among hybrids. For DK747MG and DK190MG, RE mainly determined KNP, but for the DK747RR and DK747MGRR higher PIcp had positive impact on KNP. These results highlight the importance of including secondary traits associated to KNP in breeding programs.

Introduction

Maize (*Zea mays*, L.) cultivated area in the world covers a wide range of latitudes, from 58° North in Canada to 40° South in Argentina and Chile (Fischer et al., 2014). Within this wide area, productive maize regions are characterized by average temperatures during the growing season ranging from 13°C to 32°C and mean annual rainfalls from 500 mm to 2000 mm (Bunting et al., 1982). Hence, maize crop is cultivated in humid tropical regions, humid temperate regions, semi-arid temperate regions and humid cold regions (Paliwal et al., 2001). Accordingly, under these various environmental conditions different agronomic practices were adopted to achieve economically valuable maize yields. For example, plant population density is commonly reduced under more restrictive environments (i.e. low yielding environments) while a positive grain yield response to increased plant density is obtained in high potential environments, i.e. areas with few restrictions for maize cultivation (Karlen and Camp, 1985; Popp et al., 2006; Berzsenyi and Tokatlidis, 2012). Particularly in Argentina, high yields of rainfed maize crop are obtained in the more productive regions through early sowings (late winter or early spring) and plant densities higher than 7-8 plants m^{-2}. Under more limiting environments, summer sowing with plant densities lower than 7 plants m^{-2} is the most common

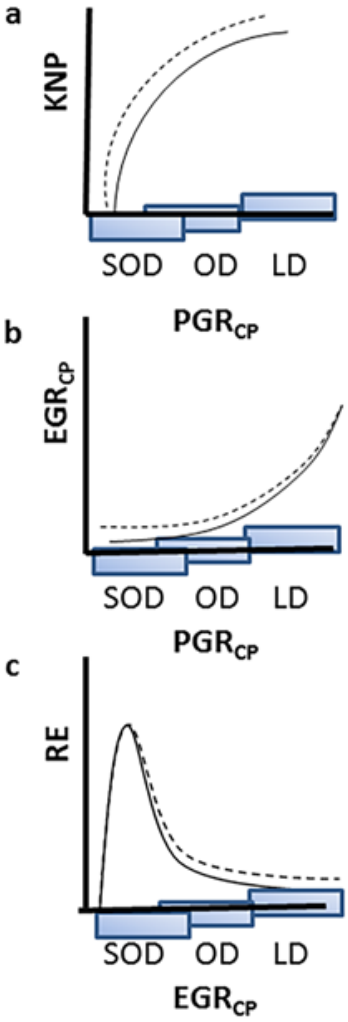

Fig. 1 - Theoretical relationships between (a) kernel number per plant (KNP) and plant growth rate during the critical period (PGR_{CP}), (b) ear growth rate during the critical period (EGR_{CP}) and PGR_{CP} and (c) reproductive efficiency of the ear (RE) and EGR_{CP} in maize crops. Rectangles on x axis identify the expected ranges of values recorded on maize plants cultivated at three contrasting plant densities (LD: low density; OD: optimum density; SOD: supra-optimum density). Solid and dashed lines represent hypothetical relationships for different genotypes

model for maize cultivation (RetAA, 2016). Despite the different sowing dates and environments, more than 90% of the cultivated maize hybrids in Argentina have single or double-stacked transgenic events to prevent Lepidoptera damage (-Bt) and to tolerate the glyphosate herbicide (-RR).

At each plant density, maize grain yield varies as a function of grain yield per plant, which is mostly determined by the number of kernels per plant (KNP). This grain yield component is related to plant growth rate (PGR) around flowering (*i.e.* the critical period; CP), biomass partitioning to the ear during this period (PI), and the efficiency of the ear to set kernels (RE, Andrade et al., 1999). Theoretical curves representing these relationships are presented in Fig 1. At low plant densities, maize plants grow with high availability of resources, both plant growth rates (PGR_{CP}) and ear growth rate (EGR_{CP}) during the critical period are close to maximum values, and plants exhibit a high biomass partitioning index (PI_{CP}) to the ears (i.e. high EGR_{CP} PGR_{CP}^{-1}) (Fig. 1a, b). However, the efficiency of the ear to set kernels (*i.e.* KNP EGR_{CP}^{-1}), or reproductive efficiency (RE), is very low (Fig. 1c) in non-prolific hybrids (*i.e.* only one ear per plant), but this decrease of RE is over-compensated by the high EGR_{CP}, yielding maximum KNP. Conversely, at higher plant densities, the availability of resources per plant is restricted, PGR_{CP} and PI_{CP} decrease (Fig. 1a, b) and RE is close to maximum value (at optimum plant density) or may drastically decrease (at supra-optimum plant density) (Fig. 1c), determining moderate or high reductions of KNP (Vega et al, 2001a); respectively. The existence of genotypic variation in these relationships was documented between older and newer maize hybrids (Echarte et al., 2004; Luque et al., 2006; Ciancio et al., 2016). Thus, at high densities differences among hybrids in KNP were associated with their different PI_{CP} (see solid vs dashed lines in Fig.1a, b). At low density, the greater KNP of some genotypes was determined by their higher RE (see solid vs dashed lines of Fig.1a, c). Phenotypic variations of these traits among non-transgenic and transgenic maize hybrids (Bt, RR, and Bt-RR versions) were documented (Laserna et al., 2012). For example, the highest EGR_{CPs} were recorded for the double-stacked versions (Bt-RR) of two different genetic backgrounds (DK747 and DK190). Moreover, the RE was not the same among versions of the same genetic background: the lowest RE values were recorded for the Bt-RR version of DK747 at high density and for the Bt-RR version of DK190 at low density. The analysis showed that some traits were compensated by others to achieve final KNP, meaning that KNP was defined by multiple secondary traits (Laserna et al., 2012). Hence, those data are useful to estimate the incidence of each trait on the definition of KNP at different genotype x plant density combinations. Additionally, a re-analysis for establishing secondary traits which define KNP at contrasting plant densities, could be helpful for breeding programs in the selection of genotypes for target environments.

The objective of this study was to identify the contribution of PGR_{CP}, PI_{CP}, EGR_{CP} and RE to KNP in six modern single-cross transgenic hybrids: the simple and staked transgenic versions of DK747 and DK190 (Bt: 747MG and 190MG; RR: 747RR and 190RR; and

Bt-RR: 747MGRR and 190MGRR) cultivated at low and high plant densities. We hypothesized that at low plant density differences among plants in KNP are mainly determined by RE. In contrast, at high plant density, differences among plants in KNP are mainly determined by PI_{CP}, but the different versions of a single-cross maize hybrid have variable strategies (i.e. combination of secondary traits) to achieve a similar KNP.

Materials and Methods

Experimental trials: design and maize genetic materials

Two field experiments were conducted in the experimental site of the Faculty of Agronomy of the University of Buenos Aires, Buenos Aires, Argentina (34 ° 35 'S, 58 ° 29' O). Experiments were carried out during 2008-2009 (mean temperature 24.9°C; cumulative incident photosynthetically active radiation 1495.2 MJ m^{-2}) and 2009-2010 (mean temperature 23.7°C; cumulative incident photosynthetically active solar radiation 1214.5 MJ m^{-2}). Tested genotypes were temperate single-cross maize hybrids produced by Monsanto Argentina, composed by the simple and staked transgenic versions of DK747 and DK190 (Bt: 747MG and 190MG; RR: 747RR and 190RR; and Bt-RR: 747MGRR and 190MGRR), hereinafter named the DK747 group and DK190 group; respectively. Hybrids were selected because of their wide use in Argentina and their contrasting adaptability (Finlay and Wilkinson, 1963): the DK747 is recommended for high potential environments and the DK190 is used for its good performance at low yielding environments and the stability of grain yields to environment variations (Ferreyra pers. comm.). Experiments were irrigated and fertilized with N, and weeds, pests and diseases were adequately controlled. Treatments were evaluated using a split plot design arranged in a randomized complete block with three replicates. The plant population density was assigned to the main plot

Table 1 - Descriptive statistics (mean, minimum and maximum values) of plant (PGRCP/gd^{-1}) and ear growth rate (EGR_{CP}) during the critical period, biomass partitioning index during this period (PI_{CP}), kernel number per plant (KNP/k) and reproductive efficiency of the ear (RE/kdg^{-1}) of two group of hybrids (DK747 and DK190) cultivated at low (D6) and high density (D12). MG, RR and MG-RR identify transgenic versions of each group of hybrids with resistance to Lepidoptera genera, glyphosate and Lepidoptera genera +glyphosate; respectively. N indicates the number of plants analyzed.

		DK747MG		DK747RR		DK747MGRR		DK190MG		DK190RR		DK190MGRR	
		D6	D12	D6	D12	D6	D12	D6	D12	D6	D12	D6	D12
PGR_{CP}	Mean	6.91	4.57	7.59	4.76	7.44	4.83	6.65	3.92	6.13	3.79	6.58	4.41
	Min	3.85	2.47	6.13	2.77	5.42	0.59	4.11	2.29	4.34	2.44	2.99	2.20
	Max	9.97	6.31	12.30	6.61	11.03	7.63	10.37	7.01	10.76	5.45	10.63	5.98
	N	59	60	58	60	60	60	60	60	60	60	60	60
EGR_{CP}	Mean	2.05	1.45	2.42	1.67	2.56	1.89	2.32	1.63	1.94	1.,48	2.57	1.97
	Min	1,31	0.78	1,47	0.71	1,95	1.02	1.23	0.49	1.35	1.11	1.63	0.99
	Max	3,50	2.24	4.12	2.68	3,14	2.72	3.84	2.24	3.38	2.40	3.19	2.72
	N	59	60	58	60	60	60	60	60	60	60	60	60
PI_{CP}	Mean	0.30	0.32	0.32	0.36	0.35	0.40	0.36	0.41	0.33	0.39	0.40	0.46
	Min	0.17	0.21	0.21	0.21	0.25	0.27	0.23	0.23	0.19	0.26	0.19	0.29
	Max	0.58	0.61	0.51	0.58	0.53	0.60	0.61	0.81	0.53	0.59	0.94	0.79
	N	59	60	58	60	60	60	60	60	60	60	60	60
KNP	Mean	653.75	465.35	668.71	461.40	653.57	455.85	760.1	438.97	782.47	454.02	776.57	458.05
	Min	542	260	677	239	520	174	513	224	442	327	518	256
	Max	872	623	850	610	890	594	1038	584	1057	620	1072	591
	N	59	60	58	60	60	60	60	60	60	60	60	60
RE	Mean	336.53	362.28	282.95	285.5	256.78	244.38	287.73	301.81	261.37	262.54	248.99	260.86
	Min	201.81	178.83	141.14	187.94	203.18	126.21	143.07	106.14	175.35	175.51	109.58	171.09
	Max	498.80	597.22	435.73	481.11	316.61	352.43	453.37	516.54	393.03	396.59	348.26	374.40
	N	59	60	58	60	60	60	60	60	60	60	60	60

Table 2 - Correlations values (r) among secondary traits (see abbreviations in Table 1) of two groups of hybrids (DK747 and DK190) cultivated at low (D6) and high density (D12). Correlation significances are indicated as + p< 0.1; * p<0.05; ** p< 0.01; *** p< 0.001.

DK747MG-D6	PGR_{CP}	EGR_{CP}	PI_{CP}	KNP	RE	DK747MG-D12	PGR_{CP}	EGR_{CP}	PI_{CP}	KNP	RE
PGR_{CP}		Ns	***	+	ns	PGR_{CP}		+	***	***	ns
EGR_{CP}	0,13		***	*	***	EGR_{CP}	0,23		***	***	***
PI_{CP}	-0,58	0,69		0,8	***	PI_{CP}	-0,59	0,62		ns	***
KNP	0,25	0,26	0,03		ns	KNP	0,5	0,52	-0,01		*
RE	-0,07	-0,91	-0,67	0,03		RE	0,13	-0,66	-0,66	0,27	
DK747RR-D6						**DK747RR-D12**					
PGR_{CP}		***	*	ns	**	PGR_{CP}		***	**	***	ns
EGR_{CP}	0,48		***	ns	***	EGR_{CP}	0,53		***	***	***
PI_{CP}	-0,28	0,7		ns	***	PI_{CP}	-0,37	0,58		ns	***
KNP	0,15	0,04	-0,08		**	KNP	0,57	0,58	0,05		*
RE	-0,36	-0,9	-0,7	0,32		RE	-0,09	-0,6	-0,62	0,27	
DK747MGRR-D6						**DK747MGRR-D12**					
PGR_{CP}		*	***	*	ns	PGR_{CP}		***	***	***	ns
EGR_{CP}	0,3		*	*	***	EGR_{CP}	0,8		ns	***	***
PI_{CP}	-0,81	0,28		ns	*	PI_{CP}	-0,57	0,01		**	***
KNP	0,28	0,26	-0,1		**	KNP	0,78	0,71	-0,39		**
RE	-0,08	-0,69	-0,3	0,38		RE	-0,07	-0,41	-0,52	0,32	
DK190MG-D6						**DK190MG-D12**					
PGR_{CP}		**	***	***	**	PGR_{CP}		***	***	***	ns
EGR_{CP}	0,40		**	+	***	EGR_{CP}	0,47		**	+	***
PI_{CP}	-0,69	0,35		*	*	PI_{CP}	-0,57	0,40		**	***
KNP	0,46	0,24	-0,31		ns	KNP	0,51	0,24	-0,35		***
RE	-0,34	-0,8	-0,27	-0,19		RE	-0,07	-0,6	-0,54	0,55	
DK190RR-D6						**DK190RR-D12**					
PGR_{CP}		***	***	**	**	PGR_{CP}		***	**	***	ns
EGR_{CP}	0,57		***	*	***	EGR_{CP}	0,56		***	***	**
PI_{CP}	-0,48	0,43		ns	***	PI_{CP}	-0,38	0,53		ns	**
KNP	0,4	0,26	-0,16		ns	KNP	0,53	0,67	0,19		**
RE	-0,38	-0,82	-0,49	-0,09		RE	-0,06	-0,39	-0,38	0,39	
DK190MGRR-D6						**DK190MGRR-D12**					
PGR_{CP}		ns	***	**	ns	PGR_{CP}		***	***	***	ns
EGR_{CP}	0,1		**	ns	***	EGR_{CP}	0,62		**	***	***
PI_{CP}	-0,78	0,37		**	**	PI_{CP}	-0,53	0,32		*	***
KNP	0,37	-0,18	-0,36		ns	KNP	0,72	0,58	-0,26		*
RE	$1,2.\ 10^{-3}$	-0,74	-0,34	0,10		RE	-0,08	-0,61	-0,56	0,27	

(D6: 6 pl m^{-2}; D12: 12 pl m^{-2}) and hybrids to the subplot (hereinafter referred as plot). Low and high plant densities were defined based on the knowledge of optimal densities for modern maize hybrids (Hernández et al., 2014) and that at D12 crowding stress is evident (Rossini et al., 2011).

Plant measurements and kernel setting secondary traits

Ten plants were tagged in the central row of each plot in order to characterize the evolution of plant biomass along the cycle, with a non-destructive technique based on measurements of morphometric variables (Vega et al., 2001b; Maddonni and Otegui, 2004; Pagano and

Maddonni, 2007; Rossini et al., 2011). For more details of this technique see Laserna et al. (2012).

Plant growth rate (PGR_{CP}) and ear growth rate (EGR_{CP}) around female flowering (i.e. -15 d and +15 d from silking date of each plant) were calculated using estimated biomass values. The biomass partitioning index to the ear during the critical period (PI_{CP}) was determined as the ratio between EGR_{CP} and PGR_{CP}. At physiological maturity all tagged plants were harvested, individually bagged and dried (60°C) to constant weight in order to determine plant biomass, grain yield and kernel number per plant (KNP). Finally, the reproductive efficiency (RE) was calculated as the ratio between KNP and the EGR_{CP}.

Statistical data analysis

Descriptive statistics (mean, maximum and minimum values) of PGR_{CP}, EGR_{CP}, PI_{CP}, KNP and RE were estimated for each genotype x plant density combination (Table 1). Correlations among these traits were obtained using Pearson's coefficient (Table 2). Principal component analysis (PCA) was used to study relationships among traits and to identify differences among plant densities, hybrids and individual plants within each group that displayed low similarity levels and reflected diversity within plant density. Data were normalized (*i.e.* each data set of Exp.1 and Exp.2 were set at mean of zero and standard deviation of one) in order to eliminate the year effect and different units of the traits.

Subsequently, cluster analysis was used to describe the structure of data by distributing the plants in classes, where the contribution of secondary traits to each cluster was determined. The proximity measure used to define the structure of the plant data set was the Euclidean distance (geometric distance in a multidimensional space), defined by Eq.1. The Euclidean distance represents the distance of each plant to the corresponding centroid. The method used was k-means (Jain and Dubes, 1988), which firstly defines k groups with a centroid (i.e. a vector containing the means for each class of the variables under study), and plants are assigned to the closer centroid by an iterative process. The final product is a clustering in which the variation among groups results higher than the variation within groups, so that each class groups those plants whose similarity in their secondary traits is maximum (Catena et al., 2003).

$$dij=\sqrt{\sum_{k=1}^{p}(xij-xjk)^2} \quad (eq^1)$$

Where d_{ij} is the geometric distance in a multidimensional space (dimensions from k=1 to k= p) between two vectors i and j.

Table 3 - Mean values of secondary traits (PGR_{CP}, EGR_{CP}, PI_{CP}, KNP, RE) of plants of two group of hybrids (DK747 and DK190), gathered at three clusters described in Figure 5

		Cluster 1	Cluster 2	Cluster 3
DK747 GROUP	PGR_{CP} (g d^{-1})	6.09	4.44	7.68
	EGR_{CP} (g d^{-1})	2.51	1,48	2.11
	PI_{CP}	0.42	0.34	0.28
	KNP (k)	595.42	439.61	666.49
	RE (k d g^{-1})	241.29	316.91	322.19
DK190 GROUP	PGR_{CP} (g d^{-1})	4.36	3.92	6.82
	EGR_{CP} (g d^{-1})	1.53	1.78	2.41
	PI_{CP}	0.35	0.45	0.36
	KNP (k)	545	450	800
	RE (k d g^{-1})	331.5	250.1	255.8

The rate of misclassification was used to define the final number of clusters that best explained the pooling of plants, by assuming that, the optimal number of clusters is the one which shows a rate of misclassification < 5% (StatSoft Inc, 2011). The rate of misclassification (*i.e.* proportion of errors during the classification procedure of plants to each cluster) was calculated as the average distance of plants to the centroid of each cluster to which they were assigned.

Results and discussion

In view of the future growing demands of agricultural products to be used as feed, food and biofuels (Andrade, 2016), breeders used different approaches to increase the annual yield rates per unit area of the main grain crops (Hall and Richards, 2013). One approach is to study the gaps between potential and actual yields under different environments in order to identify limiting factors and to define future breeding objectives (Fischer et al., 2014). For example, the use of transgenic hybrids may contribute to reduce the gap between potential and actual grain yields promoted by biotic limiting factors. Traditional breeding, however, presents limitations when it is applied to improve the adaptation of genotypes to suboptimal environments (e.g. affected by droughts), because the high intra-site variability negatively affects heritability of grain yield (Richards, 1996; Araus et al., 2002). The efforts needed in these environments to sustain the levels of genetic gain achieved in better environments, make the selection process difficult (Bäenziger Berzsenyi et al., 2006). One strategy to reach greater efficiency in these conditions is the indirect selection, which includes the measurement of secondary traits. The chosen traits should be strongly related to grain yield (Blum, 1988) or to an improved adaptability in stressful environments (Araus et al., 2008). Particularly for simple and stacked transgenic maize hybrids, Laserna et al. (2012) had

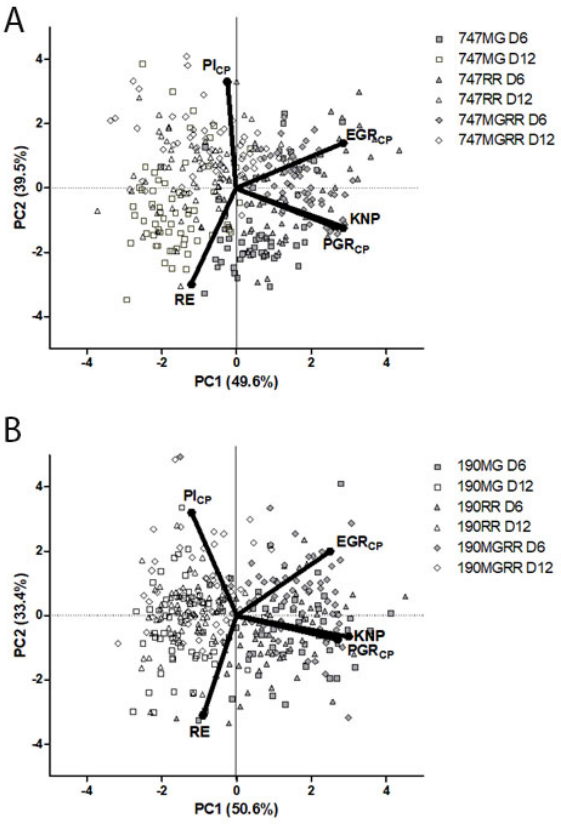

Fig. 2 - Principal component analysis for ordering maize plants of the different versions of the DK747 group (a) and the DK190 group (b) by secondary traits (PGR_{CP}, EGR_{CP}, KNP, PI_{CP} and RE) cultivated at low (D6) and high (D12) density. The size of each arrow represents the weight of each trait. Percentage of variation explained by each principal component axis (PC) is showed between brackets.

shown that for two groups of hybrids (i.e. DK747 and DK190), KNP, the main grain yield component, was defined by multiple traits, depending on the different growing conditions. By using multivariate analysis we could study the secondary traits related to KNP.

Principal Components Analysis and correlations

The PCA accounted for 89.1% and 84% of the total variance of the DK747 group and the DK190 group; respectively (Fig. 2). For both groups of hybrids, in the first PC axis (which accounted for 49.6% and 50.6% of the total variance of the DK747 group and the DK190 group; respectively), the KNP and PGR_{CP} exhibited the highest weighting and were positively and strongly associated (the vectors of these traits overlap), as documented by Tamagno et al. (2015) in other single cross hybrids. This ordering tended to separate plants along PC1, emphasizing PGR_{CP} and KNP as discriminatory variables among densities. Thus, plants at D6 (bold symbols) with high PGR_{CP} and KNP were associated with positive values of PC1. By contrast, the opposite association with PC1 was observed for plants at D12 (empty symbols). For the DK747 group, the EGR_{CP} also presented a great weight on the PC1, and together with the other two traits determined the dispersion among plants of the DK747 group on this axis (Fig. 2a).

On the other hand, PI_{CP} and RE were the traits with the heaviest weight on PC2 (which accounted for 39.5% and 33.4% of the total variance of the DK747 group and the DK190 group; respectively) and the ordering of plants along this axis was triggered by the documented contrast between these traits (Vega et al. 2001a) (Fig. 1b, c and Table 2), separating plants within each density with greater weight of PI_{CP} for the DK747 group and of RE for the DK190 group and generating a slightly greater dispersion of plants of the DK190 group along PC2.

Dispersion of plants along PC1 and PC2 axes could be indicative of the stability of hybrids in contrasting environments (Maddonni et al., 1999; Di Matteo et al., 2016) (dispersion on PC1) and/or the population variability of each hybrid within the same environment (Maddonni and Otegui, 2004) (dispersion over PC2), negatively affecting grain yield per unit area (Tollenaar and Wu, 1999; Vega et al., 2003, Di Matteo et al., 2016). Correlation analysis among traits (Table 2) supported the ordering of plants in the bi-plots but added information for each genotype x plant density combination.

For the DK747 group at D6 significant ($p<0.05$) positive correlations between KNP and some secondary traits were found for the DK747MGRR (KNP vs PGR_{CP} and KNP vs EGR_{CP}) and the DK747MG (KNP vs EGR_{CP}). Significant strong negative correlations ($r>-0.60$) were found between: i) EGR_{CP} and RE for all versions, ii) PI_{CP} and RE for the DK747MG and DK747RR, and iii) PGR_{CP} and PI_{CP} for the DK747MGRR. A positive and strong correlation ($r>0.60$) between EGR_{CP} and PI_{CP} was also found for the DK747MG and the DK747RR.

For the DK747 group at D12 significant positive correlations were found between i) KNP and PGR_{CP} and ii) KNP and EGR_{CP} for all versions and between KNP and PICP for the DK747MGRR. For the DK747MGRR strong positive correlations ($r>0.60$) were found between i) PGR_{CP} and EGR_{CP}, ii) PGR_{CP} and KNP and iii) EGR_{CP} and KNP. Significant and strong negative correlations ($r>-0.60$) were found between i) RE and EGR_{CP} for DK747MG and DK747RR and ii) RE and PI_{CP} for DK747MG and DK747RR.

All genotypes of the DK190 group at D6 showed a positive correlation between KNP and PGR_{CP} and a

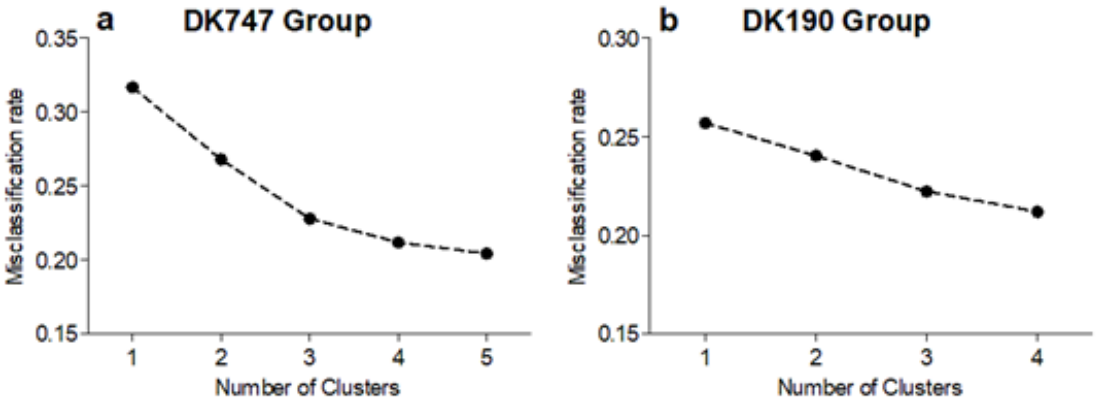

Fig. 3 - Relationship between rate of misclassification and number of clusters obtained with the analysis performed on the data sets of the DK747 group (a) and the DK190 group (b).

significant strong negative correlation (r>-0.60) between EGR_{CP} and RE. For DK190MG and DK190MGRR, PI_{CP} exhibited a strong negative correlation (r>-0.60) with PGR_{CP} (Table 2). At D12 a strong negative correlation (r>-0.60) was found between EGR_{CP} and RE for the DK190MG and DK190MGRR and a strong positive correlation (r>0.60) between EGR_{CP} and KNP for the DK190RR. For the DK190MGRR, a strong positive correlation (r>0.60) was also recorded between PGR_{CP} and EGR_{CP}.

Cluster analysis

The different population variability observed in both groups of hybrids at each plant density could be inferred from the grouping of plants by similarity of traits. The cluster analysis performed with data set of the DK747 group defined five clusters; however, rate of misclassification was less than 5% with three clusters (Fig. 3a). For the DK190 group, cluster analysis revealed four classes, but rate of misclassification was less than 5% with three clusters (Fig. 3b). Plant density had different representativeness within each class. For the DK747 group, cluster 2 was mainly composed by plants at D12, cluster 3 gathered mainly plants at D6 and cluster 1 had a slightly higher percentage of plants at D6 than at D12 (Fig. 4a). For the DK190 group, cluster 1 had a higher percentage of plants at D12 than at D6, cluster 2 was mainly composed by plants at D12 and cluster 3 mainly gathered plants at D6 (Fig. 4b).

Considering cluster classification of plants (Fig 5), at low plant density each group of hybrids presented a cluster in which the highest percentage of plants showed the highest PGR_{CP} and KNP (cluster 3 for both group of hybrids). Some plants at low plant density (Fig. 4; ca. 60% for the DK747 group and ca. 30% for the DK190 group), however, were also grouped in clusters with mid values of KNP given by both intermediate PGR_{CP} and high PI_{CP} (cluster 1 for DK747 group, Table 3, Fig. 5a) or high RE (cluster 1 for DK190 group, Table 3, Fig. 5b). These plants would be the most suppressed individuals of the

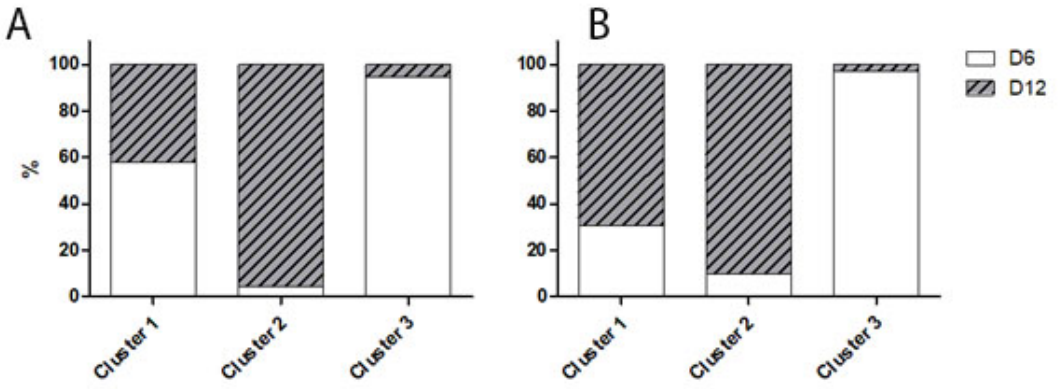

Fig. 4 - Percentage of maize plants cultivated at low densities (D6 and D12) classified in three clusters obtained with the analysis performed on the data sets of the DK747 group (a) and the DK190 group (b).

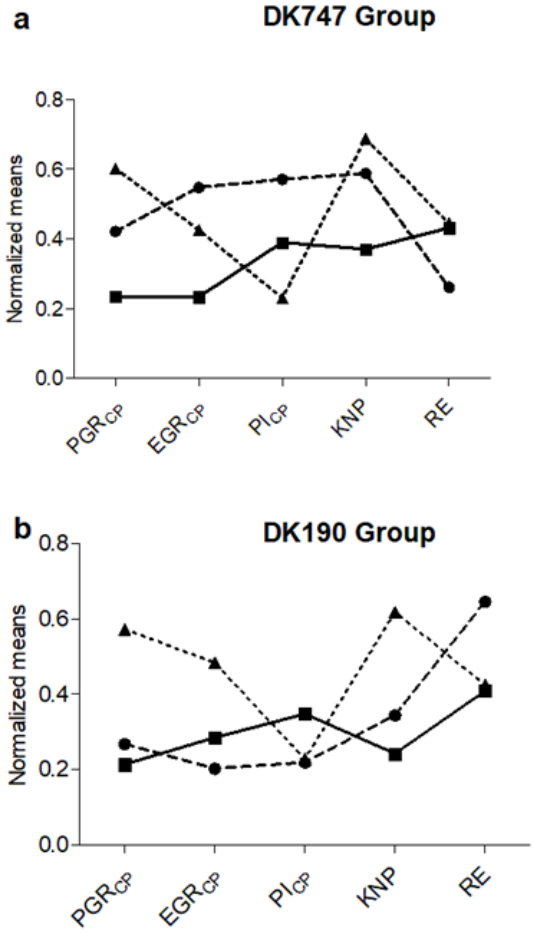

Fig. 5 - Normalized mean values of secondary traits at each cluster, for DK747 group (a) and DK190 group (b). For each trait normalization was carried out by considering a mean of zero and a standard error of 1 in order to compare at each cluster, the weight of traits measured with different units (circles: cluster 1; squares: cluster 2; triangles: cluster3).

stand at this low density, which partially compensated the reduction in their growth during the critical period (i.e. intermediate PGR_{CP}) by a higher PI_{CP} or RE (see solid vs dotted lines in Fig. 1 b and c). A positive impact of RE on KNP was previously documented by Echarte et al. (2004) analyzing older and modern maize hybrids at low density, but the importance of PI_{CP} at low density was never documented. Therefore, PGR_{CP} was a critical trait to determine KNP at low planting density but PI_{CP} or RE also contributed to KNP of the DK747 group and the D190 group, respectively, which allows the rejection of the first hypothesis (at low plant density differences among plants in KNP are mainly determined by RE).

At high plant density, plants were mainly grouped in one cluster (cluster 2 for both group of hybrids), characterized by plants with low KNP and PGR_{CP} (Table 3, Fig. 5). However, some plants were gathered in other clusters and it was a differential behavior in the grouping of the plants that belong to different versions (Fig. 6). These clusters were mainly determined by the high PI_{CP} (cluster 1 for DK747 group, Fig. 5a) or RE (cluster 1 for the DK190 group, Fig. 5b) also shared by plants of the low density. Hence, the classification of plant hierarchies within a genotype at a particular density using different traits would be more functional than a classification only based on plant biomass (Maddonni and Otegui, 2004), since it allows to observe positive or negative compensations among traits that define KNP. Thus, at high density some plants with low PGR_{CP} achieved higher KNP by their increased PI_{CP} or RE, explaining the observed decoupling between plant growth around flowering and the reproductive success of plants of a high-density maize canopy (Vega and Sadras 2003). The physiological basis of the change in these traits is still uncertain.

Finally, the percentage of plants of the different versions within clusters was estimated for each combination of group of hybrids and plant densities. For both groups of hybrids at D6, the highest percentage of plants of all versions was gathered in cluster 3 (ca. 60% for the DK747 group, Fig. 6a; and ca. >80% for the 190 Group, Fig. 6b). Plants of this cluster presented the highest KNP and PGR_{CP} (Table 3).

At high plant density in both groups of hybrids, the three

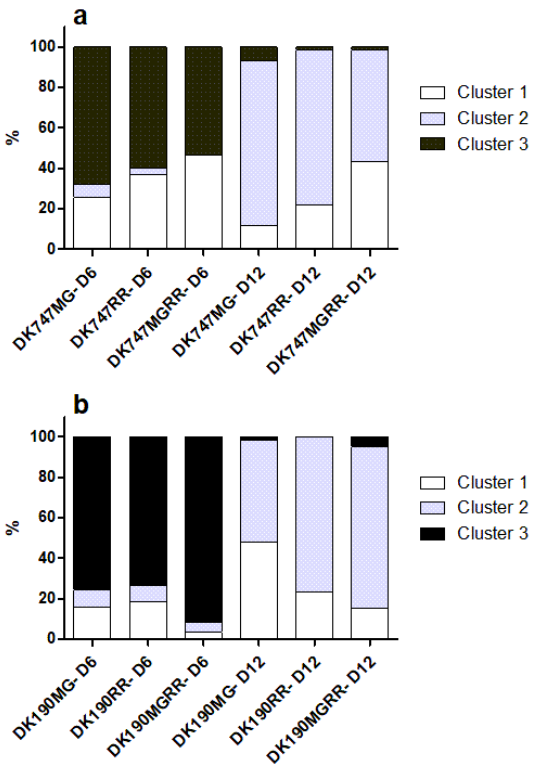

Fig. 6 - Percentage of maize plants of the different versions of the DK747 group (a) and DK190 group (b) cultivated at two densities (D6 and D12) classified within the three clusters described in Fig. 4 and 5.

versions showed differences in the proportion of plants in cluster 1 (high EGR_{CP} and PI_{CP} for DK747 group and high RE for DK190 group) and 2 (low PGR_{CP}, EGR_{CP} and higher RE). Plants of the DK747MG and DK747RR were mainly those (ca. 81.67% and 76.67%, respectively) of cluster 2 with the lowest values of KNP, PGR_{CP} and EGR_{CP} but high RE (Table 3). The DK747MGRR plants were similarly represented in cluster 2 (ca. 53.33%) and 1 (ca. 43.67%), with intermediate values of KNP and high of EGR_{CP} and the highest value of PI_{CP} (Table 3). For the DK190 group, cluster 2 grouped most plants of DK190RR and DK190MGRR and half of the plants of DK190MG (plants with low PGR_{CP} and KNP but the highest PI_{CP} ; Table 3) (Fig. 6b). The rest of the plants of these hybrids were gathered in cluster 1, with intermediate KNP and the highest RE (Table 3).

The increment of the DK747RR and DK747MGRR plants representing the cluster 1 with respect to the DK747MG version, means that the greater KNP of DK747RR and DK747MGRR at high plant density (i.e., their higher tolerance to crowding) was determined by a high PI_{CP}, which could be related to the incorporation of the simple or stacked -RR event (through the transgene or through the process of line conversion), although it was not possible to find a molecular evidence (Laserna et al., 2015). In contrast, for these transgenic versions of the DK190 group, a high PI_{CP} did not compensate for the low PGR_{CP}. These results demonstrate that the observations of Echarte et al. (2004) on the positive impact of PI_{CP} in KNP at high plant density does not appear to be reflected in a better kernel setting of DK190RR and DK190MGRR but can be observed in DK747RR and DK747MGRR. Consequently, second hypothesis is partially accepted (at high plant density, differences among plants in KNP are mainly determined by PI_{CP} and the different versions of a single cross maize hybrid have variable strategies, i.e. combination of traits, to achieve a similar KNP).

Conclusions

In this work, we highlighted the importance of secondary traits associated with kernel setting in two groups (DK747 and DK190) of single-cross maize transgenic hybrids. The incorporation of these traits in breeding programs is the final aim to sustain and increase genetic gains for yield. At low plant density, PGR_{CP} was the critical trait for kernel setting in both groups of hybrids, but PI_{CP} or RE also contributed to KNP of the DK747 group and the D190 group; respectively. At high plant density, a differential contribution of secondary traits on KNP was recorded among hybrids. For the DK747MG RE mainly determined KNP, but for the DK747RR and especially for the DK747MGRR an increased PI_{CP} had a positive impact on KNP. Similarly, RE had a positive impact on the determination of the KNP for the DK190MG while it was lower for the other two versions.

However, measuring changes in secondary traits of interest among genotypes establish a new challenge for physiologists and breeders since measurements of these traits, are time consuming, making difficult their implementation in breeding programs (Otegui et al., 2015). The use of molecular markers associated to the secondary traits and the increasing use of crop phenotyping platforms could help breeders to make use of important secondary traits in the near future. New genotyping technologies as massive use of SNPs (Single nucleotide polymorphism) or DArTseq (Diversity arrays technology by sequencing) may help to characterize the genetic variation associated to these traits. Unfortunately, there are no QTL experiments describing molecular markers associated to these characters (maizegdb.org) although many of them may be related to QTLs for grain yield. Future experiments would explore genomic regions associated to PI_{CP} and RE specifically.

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